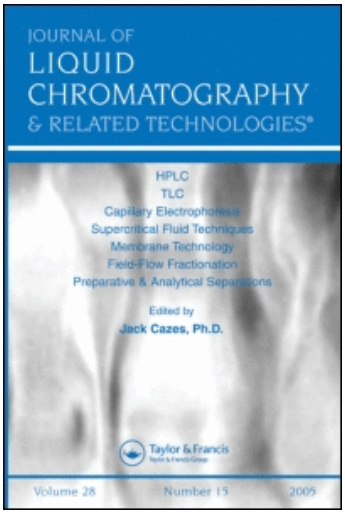

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Experimental Studies with a Bonded N-acetylaminopropylsilica Stationary Phase for the Aqueous High Performance Exclusion Chromatogrpahy of Polypeptides and Proteins

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EXPERIMENTAL STUDIES WITH A BONDED N-ACETYLAMINOPROPYLSILICA STATIONARY PHASE FOR THE AQUEOUS HIGH PERFORMANCE EXCLUSION CHROMATOGRPAHY OF POLYPEPTIDES AND PROTEINS*

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ABSTRACT

The potential of the micoparticulate, chemically bonded N-acetylaminopropylsilica stationary phase of nominal pore diameter of 100 angstroms in the high speed gel permeation chromatography of polypeptides and small proteins has been further investigated. The influence of ionic strength on the elution behaviour of a selected group of polypeptides and proteins on this bonded hydrophilic support has been examined. The results obtained with this porous, microparticulate bonded 'amide' phase silica support, packed into standard analytical-

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size stainless steel HPLC columns, indicate that milligram quantities of polypeptides and proteins with molecular mass up to 45,000-50,000 daltons can be efficiently fractionated with excellent recoveries of biological activities. The role of silica-based sorbents in the gel permeation fractionation of polypeptide and protein hormones, including those of pituitary and hypothalamic origin, is discussed.

INTRODUCTION

Gel permeation chromatography of polypeptides and proteins on agaroses, cross-linked dextrans and cross-linked polyacrylamide copolymer gel supports has been extensively used over the last two decades for their fractionation, in desalting experiments as well as for the estimation of the molecular weights of these biopolymers in their native and denatured states. Most conventional xerogels, which have been used as stationary-phases in these open column separations, are unsuited to the chromatographic conditions now commonly employed with high performance liquid chromatographic (HPLC) techniques. For example, variation in eluent composition will result in a change in the degree of swelling of conventional xerogels and this in turn will affect the column bed volume and the pore structure on which the separation depends. Deformation of the gel structure is also commonly found with many of the earlier types of soft hydrophilic organic gels at high flow rates or viscosities of the mobile phase. Recently, attention has focused on the use of pressure stable hydrophilic aerogels of small particle size as stationary phases in the high speed gel permeation HPLC of water soluble biomolecules. Because of its favourable physical and chemical properties rigid, porous silica has been favoured as the support matrix for the preparation of these hydrophilic aerogels via modification of the silica surface with an adsorptively coated or chemically bonded ligand of suitable functionality.

Porous silica itself is generally unsuited for the separation of polypeptides and proteins due to severe ion exclusion and adsorption effects. Although these effects can be partially reduced by adsorptively coating the silica surface with hydrophilic polymers such as polyethylene glycols, the resultant phases exhibit generally poor stability. By chemically bonding hydrophilic phases to accessible silanol groups on the silica surface, many of the limitations arising from the strong ionic and hydrogen bonding interactions between charged or polar groups in the protein or polypeptide and the silica surface can be largely avoided. The 1,2-dihydroxy-3-propoxy-propyl modified silicas ('glycophase'-bonded supports) developed by Regnier and Noel [2] and Becker and Unger [3,4] have been used in several studies [5-10] related to the gel permeation HPLC of native proteins. More recently, the Toya Soda TSK-Gel SW series and the Waters PAC I-series of chemically bonded silica stationary phases have been found to give satisfactory recoveries of native proteins in a number of small scale fractionation studies as well as good correlation between log M.W. and the elution volume for protein polypeptides in the presence of denaturing reagents [11-20]. To date the precise chemical and physical compositions of the bonded phases used in both of these types of commercial support remain proprietary information. The chemical modification of the surface silanol groups of silica with triethoxy-N-acetylaminopropylsilane has been shown by Engelhardt and Mathes [21-23] to yield a hydrophilic 'amide' stationary phase suitable for the exclusion chromatography of water soluble polymers, including proteins.

Most of the previous studies with chemically bonded hydrophilic silicas have been concerned more with the

analytical potential of these supports. In this paper we describe further experimental studies addressed to the small scale preparative exclusion chromatography of polypeptides and proteins using this 'amide' phase bonded to microparticulate, porous silica with a nominal pore diameter of 100 angstroms. The data obtained on the recoveries and elution characteristics of a range of polypeptides and proteins, indicates that solutes in the range 2-40 x 10^3 daltons can be successfully fractionated on this support. The feasibility of using this, and related bonded hydrophilic phase silicas, in the purification of peptide and protein hormones by a combination of gel permeation and reversed phase HPLC techniques is discussed.

MATERIALS AND METHODS

High Performance Liquid Chromatography.

The liquid chromatograph consisted of a M6000A solvent delivery unit, U6K universal injector and a M450 variable wavelength UV monitor (all from Waters Assoc., Milford, Mass., U.S.A.) and a Rikadenki dual channel chart recorder. The bonded 'amide' stationary phase was prepared from LiChrosorb Si100 as previously described [23]. This bonded 'amide' phase silica has an effective surface coverage of bound ligand of 4.4μmol/m^2, whilst the silica matrix used had an average pore diameter of 100 Å, a specific pore volume of 1cm^3/g and a specific surface area of ca. 300m^2/g. The nominal particle diameter is 10μm. All separations were carried out on a standard drilled stainless steel column (25cm x 4.1mm I.D.) slurry packed with the bonded phase. The column volume was 3.3ml. The elution volume (V_e) of each solute was determined from the elution time (V_T) multipled by flow rate, and related to the interstitial volume, V_Z and pore volume, V_p, of the

stationary phase in the column by $V_e = V_Z + \kappa V_P$ where κ is the Wheaton-Baumann distribution coefficient [24]. The sample injections (1-25µl) at protein concentrations in the range 1-20mg/ml were made with Pressure Lok liquid syringes from Precision Sampling Corp., (Baton Rouge, La. U.S.A.). Eluants were degassed and filtered as reported previously [5]. All chromatography was carried out at room temperature (ca. 18^0). The mobile phase flow rate was 0.5ml/min unless otherwise indicated in the text.

Chemicals and Reagents.

Tris buffer was obtained from Sigma Chemical Co., (St Louis, Mo., U.S.A.) orthophosphoric acid, and sodium chloride A.R. were obtained from May and Baker (Dagenham, Great Britain). The source of some of the polypeptides and proteins (Table) used in this study has been given previously [25], the remainder were either prepared and purified in this laboratory or purchased from Sigma Chemical Co., Miles Laboratories (Kankakee, Ill., U.S.A.) or Worthington Biochemical Corp., (Freehold, New Jersey, U.S.A.). The ovine anterior pituitary proteins were isolated by a new procedure to be reported elsewhere [18]. The ovine thyroid binding protein preparation was prepared as previously described [26,27] whilst the biological potency of the ovine thyrotrophin and thyroid binding protein preparations were assessed by the McKenzie mouse bioassay [28]. Protein recoveries were determined by the Bradford assay [29] and peak area integration. Detection was at 215nm.

RESULTS AND DISCUSSIONS

Over the past several years, HPLC techniques have attracted considerable attention for the purification of peptides, polypeptides and proteins from tissue homogenates.

TABLE

Polypeptide/Protein	M.W.	pI*	t_e(sec)
1. Phenylalanine	165	6.0	340
2. Tryptophan	204	5.9	367
3. Oxytocin	1,007	~6.6	289
4. Ile^5-Angiotensin II	1,046	~6.8	288
5. Adrenocorticotropin(1-24)	2,930	~8.0	280
6. Bovine Insulin	~5,800	5.3	275
7. Cytochrome C	~12,200	9.3	344
8. Lysozyme	~14,300	11.0	834
9. Myoglobin	~17,200	7.1	251
10. Bovine Growth Hormone	~23,000	7.3	239
11. Bovine Trypsin	~23,300	10.8	297
12. Chymotrypsinogen	~25,700	9.5	309
13. Ovine Thyrotrophin	~32,000	5.5-6.8	220
14. Bovine β-Lactoglobulin	~35,000	5.1	243
15. Ovalbumin	~43,000	4.7	211
16. Catalase	~58,000	6.7	184
17. Bovine Haemoglobin	~64,500	6.8	287
18. Bovine Serum Albumin	~68,000	4.4-4.8	196
19. Lactate Dehydrogenase	~144,000	5.2	194
20. Aldolase	~160,000	9.1-9.7	184
21. Ferritin	~440,000	4.2-4.5	184
22. Thyroglobulin	~660,000	4.5	181

* Data from ref. [18, 40-43].

Recent publications from our and other laboratories have clearly demonstrated that the separation and recovery of bioactive polypeptides and even small proteins up to ca 2×10^4 daltons can be achieved by reversed phase HPLC with appropriately chosen mobile phase conditions, i.e. suitable mole fraction and elutropic characteristics of the organic solvent modifier, pH buffer composition, temperature (for reviews of recent applications and elution strategies see [30-35]). With careful attention to the parameters which control the secondary chemical equilibria established between the solute molecules, the mobile phase and the stationary phase and to the role these effects play in modulating the overall chromatographic distribution process, greatly improved resolution and recoveries can be obtained on microparticulate reversed phase silicas when compared to open column gel or ion-exchange chromatographic separations. With some larger proteins, unsatisfactory resolution and recoveries have been observed with the current generation of reversed phase silicas due in part to inappropriate porosities and surface coverage of the silica support, unfavourable solubility parameter dependencies of the protein solutes on the mobile phase composition, the tendency for little, or no, elution development to occur and to the poor mass transfer kinetics exhibited by many macroglobulins. For these reasons alone, the wider use of chemically stable, non-compressible bonded hydrophilic phase silicas of appropriate pore structure as gel permeation HPLC supports would considerably supplement existing capabilities of reversed phase HPLC methods. A further strong justification for reliable gel permeation HPLC supports comes from the important requirement in most protein purification strategies and particularly with the small- or micro-scale purification of biologically potent or labile proteins, to use two or more

high resolution techniques, preferably based on different separation phenomena, as early and as rapidly in the isolation protocol as possible.

For a bonded phase support to be effective in the gel permeation HPLC separations of biopolymers, the choice of the ligand to be used in the chemical modification of the surface of the porous silica must be based on at least the following four considerations, namely (1) the derivatisation reaction should provide a dense surface coverage of the ligand which acts as a water wettable, non-ionic hydrophilic surface, (2) the ligand neither selectively adsorbs or repels proteins and ideally should be of similar polarity to that of the biopolymers to be separated, (3) the bonded ligand must be chemically stable to the buffered solutions and pH conditions commonly used for peptide or protein isolations and (4) the carbonaceous silane reagent should be easily prepared, form a stable monolayer rather than a polymeric bonded layer and have a small average place requirement. Practical constaints in the coating technology do not currently allow these requirements to be fully met. For example, it is not possible due to steric restrictions and other limitations in the derivatisation reaction to completely modify all the silanols on the silica surface. The presence of residual silanols in bonded hydrophilic supports will lead to ionic interactions most noticable with very basic (i.e. pI > 9) or very acidic (i.e. pI < 5) proteins. These electrostatic interactions can be minimised by the appropriate choice of mobile phase ionic strength or by the use of acidic amine buffers [19,31,32].

The contribution of ionic interactions between the bonded 'amide' phase and the polypeptides and proteins shown in the Table was assessed by comparing the elution volumes as a function of ionic strength at pH 7.5. Representative

results are shown in Figure 1. It was apparent from these experiments that the more basic proteins, e.g. lysozyme, cytochrome c, showed greater dependency of their elution volumes on ionic strength than the weakly basic or acidic proteins and polypeptides. At low ionic strength, negatively charged polypeptides would be expected to be partially excluded from the negatively charge pores of the stationary phase. This will lead to smaller elution volumes than expected on the basis of their molecular weights. Experimentally, this has been observed [9,22] with both the 'amide'

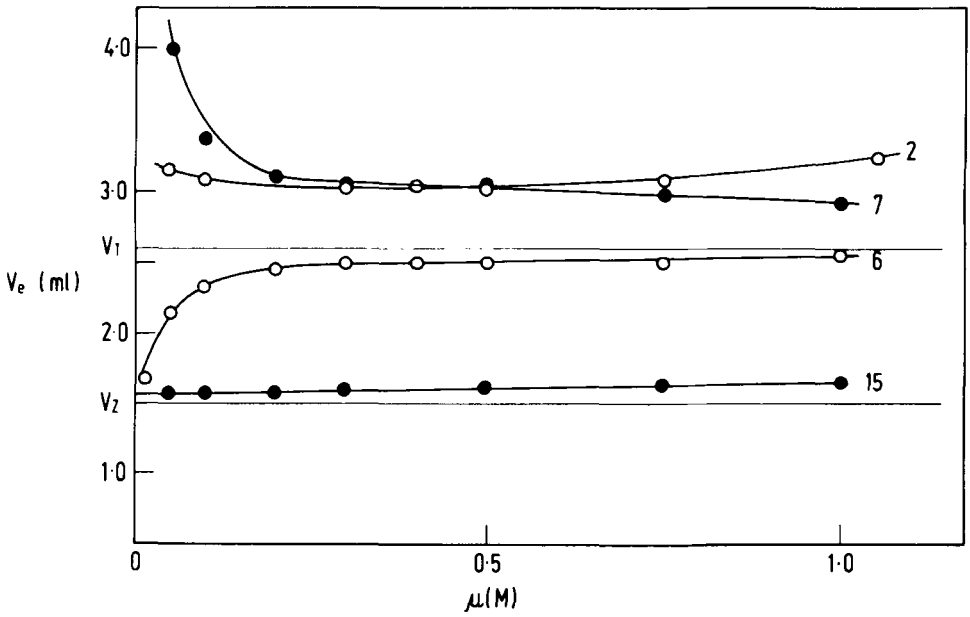

Figure 1.

Retention behaviour of several polypeptides and proteins on the bonded 'amide' phase LiChrosorb Si 100 support. The plots show the variation of the elution volume of different solutes as a function of ionic strength. See the Table for the solute key.

and the 'glyco-phase' supports. Weakly basic and weakly acidic proteins would be expected to be the least affected by changes in the ionic strength and this was generally found in the present study. For most of the peptides and proteins examined the elution volumes were essentially independent of ionic strength above μ=0.3M. For assay convenience, a 25mM Tris-HCl/125mM NaCl (pH 7.5) eluent was found most effective in several subsequent protein isolations. The same eluent was used for the investigation of the log M.W. depending on elution volume. Shown in Figure 2 and 3 are the plots of

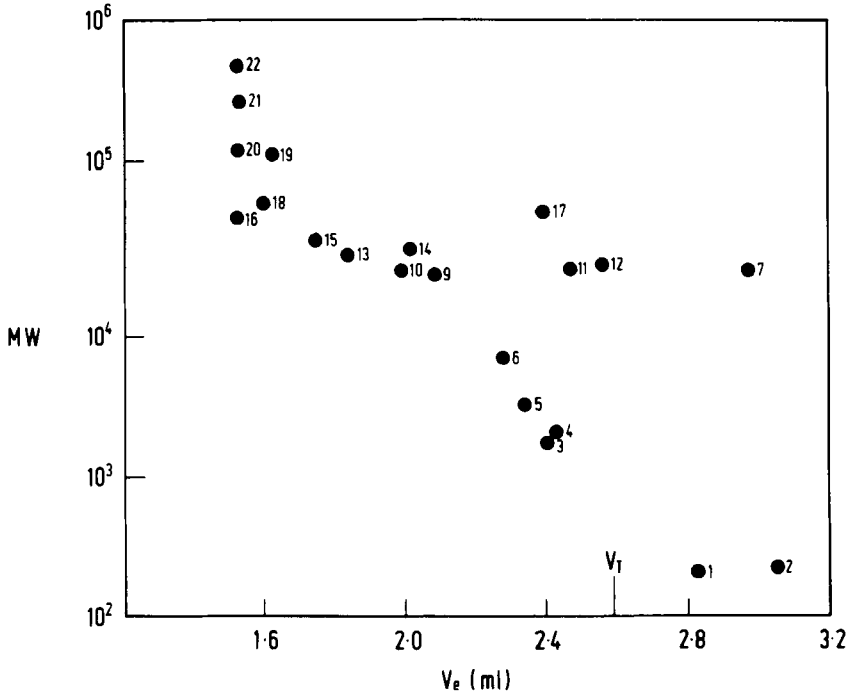

Figure 2.

Plot the log M.W. versus elution volume using a 25mM Tris-HCl/125mM NaCl (pH 7.5) eluent. The legend for the solutes is given in the Table.

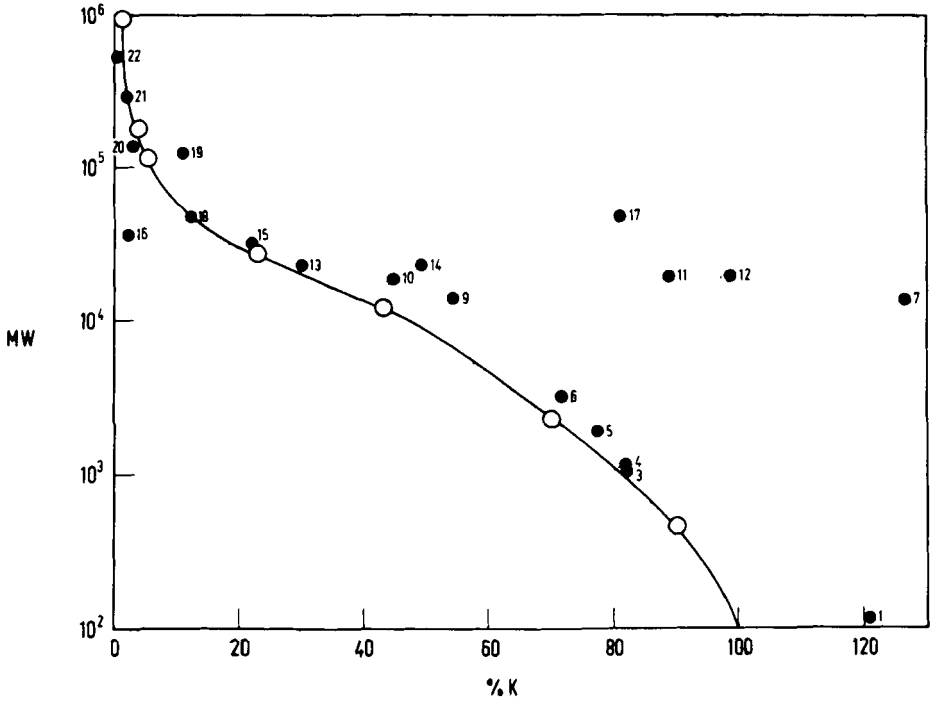

Figure 3.

Plot of the log M.W. versus percentage K of representative polypeptides and proteins. The calibration curve of polystyrene standards eluted with CH_2Cl_2 from the same hydrophilic support is also shown (-O-). Conditions as in Figure 2.

log M.W. versus V_e and log M.W. versus % K for a range of peptides and proteins determined under these conditions. Proteins with a molecular mass greater than 68,000 daltons behave as though they are essentially excluded, i.e. $K < 0.1$, whilst some low molecular weight peptides and hydrophobic proteins e.g. lysozyme, are retained beyond the theoretical internal volume (as determined with D_2O). Under high ionic strength conditions the divergencies observed in elution behaviour from those

anticipated on the basis solely of a non-ionic gel permeation phenomenon presumably reflect the participation of hydrophobic, rather than electrostatic, interactions between the stationary phase and the solutes. Similar observations have been made by Schmidt et al. [9] in their study on the chromatographic behaviour of proteins on LiChrosorb DIOL and parallel previous findings [36,37] on the relative hydrophobicities of a number of proteins on n-alkyl substituted agaroses and Spheron-300. It is noteworthy that the carbonaceous coverage of the 'amide' phase used in the present study ($4.4\mu mol/m^2$), is approximately twice the value reported [9] for the LiChrosorb DIOL phase, ($2.1\text{-}2.5\mu mol/m^2$). Additional studies have shown [21, 22,38] that improved correlation for the plot of log M.W. versus elution volume can be obtained by using the weight average molecular weight as derived from the Mark-Houwink equation [39] which relates the molecular weight of a protein to its effective Stokes radius in solution. At pH 7.5 and μ>0.3M, the recoveries for the proteins and enzymes used in this study were in the range 85-95% with no significant changes in elution behaviour with sample loadings ranging from 10-500μg on an analytical size column.

Based on the above observations with the selected range of polypeptides and proteins shown in the Table, the use of this surface modified silica as a stationary phase for the gel permeation HPLC fractionation of crude protein mixtures from tissue sources was examined. Many of the polypeptides and proteins currently of interest in, for example, endocrine and neuroendocrine research fall into the molecular weight inclusion range of this stationary phase, i.e. between 2-40 $x10^3$. In addition, most of these substances are available only in minute amounts, e.g. 1μg per gram tissue. Rapid high resolution micromethodologies are obviously needed for their

analysis and purification. When only small amounts of crude polypeptide samples, e.g. less than 10mg, are available, it should be generally feasible to now base their fractionation solely on HPLC methods. A combination of conventional open column chromatographic and HPLC techniques are usually required for reasons of economy when large amounts of crude protein preparations are available. For example, as a routine procedure in use in our laboratory with salt or solvent fractionated tissue extracts, including crude pituitary preparations, we have found that gel permeation and reversed phase HPLC methods under several elution conditions will satisfactorally fractionate ca. 1-5mg of material whilst with amounts above 50mg a sequential combination of open column gel permeation chromatography, a lectin affinity chromatography separation if the substances of interest are glycoproteins, gel permeation HPLC on this bonded 'amide'-, or alternatively the bonded glycerylpropyl- [18], support and a reversed phase HPLC separation on a 100 Å or a 500 Å alkylsilica, most efficient in terms of time, cost, homogeneity and final recovery yield of a specific bioactive polypeptide or protein. In some cases, a normal phase separation on a bonded hydrophilic phase silica using a decreasing gradient of an organic solvent modifier may also prove advantageous. Rubinstein et al. [10] have also employed this approach for the fractionation of human leucocyte interferon on a LiChrosorb DIOL support. Typical of the use of the bonded 'amide' phase as a gel permeation support are the chromatographic profiles shown in Figure 4. These chromatograms represent the high speed exclusion separation of a crude ovine thyrotrophin preparation ex the 1.4M ammonium sulphate (pH 4.0) cut and different fractions containing this glycoprotein hormone sequentially rechromatographed on the same column. After

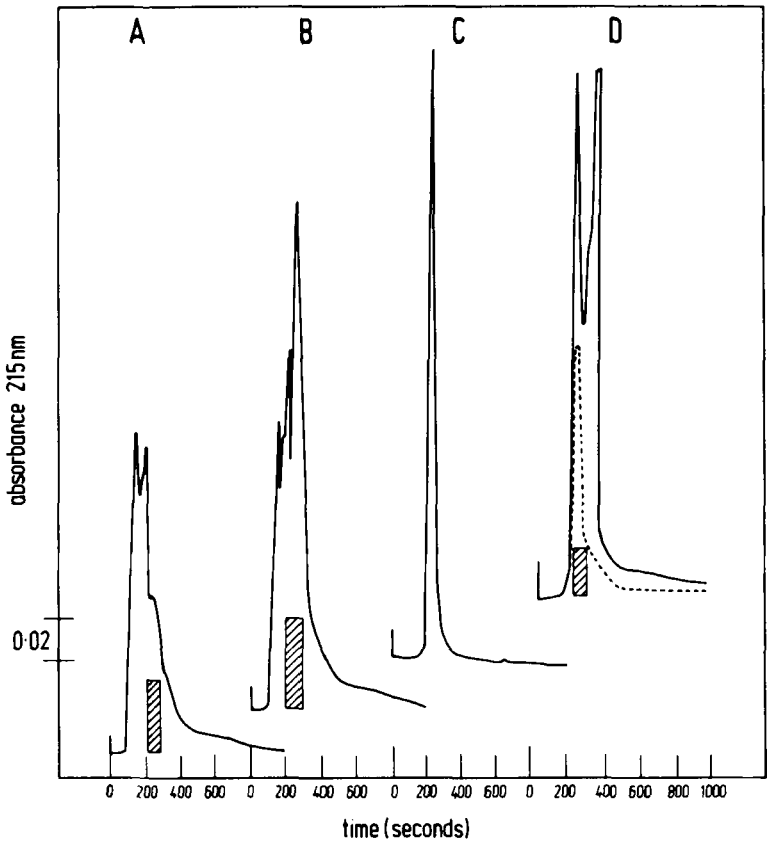

Figure 4.

Chromatography of (A) a crude ovine thyrotrophin preparation on the bonded amide phase, (B) a Sephadex G100 fractionated ovine thyrotrophin preparation, and (C) a sample of this protein after three gel permeation HPLC cycles on the bonded 'amide' phase column, flow rate, 0.5ml/min., eluent 100mM Tris HCl-500mM NaCl, pH 7.4. The regions indicated by hatched zones contained the thyrotrophic activity as assessed in the mouse bioassay. In (D) is shown the elution profiles of a 1 x chromatographed ovine thyrotrophin preparation before and after affinity chromatography on Concanavalin A-Sepharose CL4B, flow rate 0.5ml/min., eluent 25mM Tris, HCl-125mM NaCl, pH 7.4.

three elution-, collection-, desalting- and concentration-cycles, the overall recovery of bioactive ovine thyrotrophin, as assessed by the McKenzie mouse bioassay, was 83% with a total elution time for each chromatographic injection/elution/collection step of ca. 10mins. As we have shown elsewhere [18] this preparation can be used either directly, or following further purification on a 500 Å bonded octylsilica with a triethylammonium formate-acetonitrile eluent, in binding studies with thyroid plasma membrane components. Even though the sample capacity of the analytical size column used in the present study was relatively low, i.e. maximum 250-500µg/protein injection depending on the complexity of the protein mixture, when compared to conventional Sephadex G100 columns of bed volume ca 300ml previously employed for the gel filtration of crude glycoprotein hormones, including crude thyrotrophin preparations, the short elution times, excellent recoveries and improved resolution still allowed milligram quantities of this pituitary protein to be quickly fractionated. Clearly, larger sample loadings, e.g. 10-100mg/injection could be accomodated if desired, with HPLC semi-preparative or preparative columns packed with this bonded 'amide' phase support.

The bonded 'amide' phase support has also been successfully employed as the fractionation of the solubilised sheep thyroid binding protein specific for human thyroid stimulating auto-antibodies present in patients with Graves' disease (Fig.5). After 13 consecutive injections of 300µg protein/injection, no change in the resolution was evident, with a total protein recovery after desalting and lyophilisation of 3.8mg, (97%).

In conclusion, the bonded N-acetylaminopropyl-silica stationary phase of 100 Å nominal pore diameter is a useful

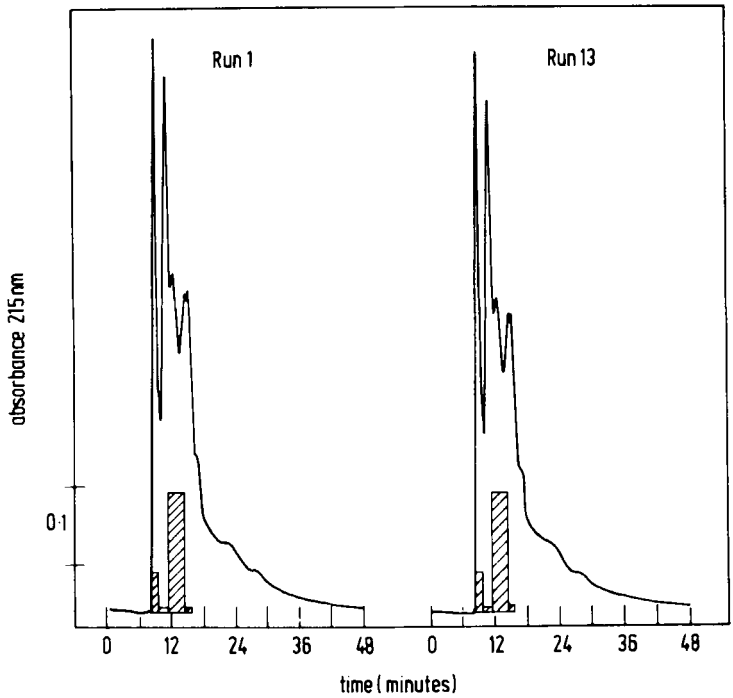

Figure 5.

Fractionation of the crude solubilised sheep thyroid binding protein components specific for human thyroid stimulating autoantibodies on the bonded 'amide'phase support. The profiles correspond to the initial loading (A) and the thirteenth consecutive loading (B) of 300µg of protein/injection. Flow rate 0.2ml/min; eluent, 100mM Tris/HCl-500mM NaCl, pH7.4. The hatched zones correspond to regions of autoantibody binding activity.

support for the aqueous size exclusion HPLC separation of polypeptides and small proteins. Similar supports of larger pore diameter, e.g. 500 Å, would be expected to extend the molecular weight range for exclusion chromatography to larger macroglobulins. Preliminary studies [38] on the chromatography of several common proteins on these large pore bonded 'amide'

phase silicas have confirmed this expectation. It is anticipated that these and related, bonded hydrophilic phase silicas will gain increasing popularity for the rapid fractionation of biologically active polypeptides and proteins by size exclusion HPLC.

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